Emerging functional roles of nuclear receptors in breast cancer

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Abstract

Nuclear receptors (NRs) have been targets of intensive drug development for decades due to their roles as key regulators of multiple developmental, physiological and disease processes. In breast cancer, expression of the estrogen and progesterone receptor remains clinically important in predicting prognosis and determining therapeutic strategies. More recently, there is growing evidence supporting the involvement of multiple nuclear receptors other than the estrogen and progesterone receptors, in the regulation of various processes important to the initiation and progression of breast cancer. We review new insights into the mechanisms of action of NRs made possible by recent advances in genomic technologies and focus on the emerging functional roles of NRs in breast cancer biology, including their involvement in circadian regulation, metabolic reprogramming and breast cancer migration and metastasis.

Key Words

- ▶ nuclear receptors
- ▶ breast cancer
- ▶ circadian clock
- ▶ metabolism
- ▶ migration and metastasis

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Introduction

Breast cancer is the most common cancer diagnosed in women worldwide (Ferlay *et al.* 2015). Over the past decades, substantial progress toward treatment of primary estrogen receptor-positive (ER+) breast cancer has been made with the development of endocrine therapies targeting the estrogen biosynthesis and signaling pathways. Despite the success of endocrine therapies, there remain subgroups of women for whom available treatment offers little help. These include patients with ER+ breast tumors who develop endocrine treatment resistance (Dixon 2014), patients with estrogen receptor-negative (ER–) breast cancers (Hudis & Gianni 2011) and cancers that recur in all age groups (EBCTCG 2005). Women in these subgroups currently face the challenge of living with advanced disease; therefore, there exists the need to develop rational treatments targeting these subgroups of breast cancer.

Nuclear receptors (NRs) are potential promising targets due to the importance of NRs as master regulators of

nearly all physiological aspects of life and the availability of drugs targeting NRs that resulted from intensive drug development targeting NRs for a range of pathological conditions. The human NR superfamily consists of 48 highly evolutionarily conserved transcription factors with the ability to act as molecular sensors of physiological and environmental stimuli (Mangelsdorf *et al.* 1995). Development of technologies that allow genome-wide, unbiased profiling of genomic binding sites and gene expression has allowed deeper insights into the molecular mechanisms of NR actions. These studies highlight the complexity of gene regulatory networks governed by NRs, the extensive nature of NR crosstalk and the role of coregulators and the chromatin landscape of the cell as important modulators of NR cell type-specific and context-dependent function.

In the context of breast cancer, it is recognized that estrogen receptor (ESR1) and progesterone receptor (PGR) play critical roles in the development and progression

of breast cancer, and moreover, their expression in a breast tumor remains the single most robust predictor of disease outcome. Consequently, measurement of *ESR1* and *PGR* expression in breast cancers is a mainstay of clinical management programs and treatments targeting the estrogen signaling axis are standard of care for ER+ disease (Cordera & Jordan 2006). More recently, the androgen receptor (AR), which is frequently expressed in primary breast tumors (Park *et al.* 2010, Grogg *et al.* 2015), has emerged both as a marker of outcome and as a potential treatment target (Cordera & Jordan 2006, Barton *et al.* 2015). A number of other nuclear receptors, including the vitamin D3 receptor (VDR) (Krishnan *et al.* 2010, Mehta *et al.* 2013) and the glucocorticoid receptor (GR) (Vilasco *et al.* 2011, Abduljabbar *et al.* 2015b) have also been investigated for their anti-proliferative and anti-apoptotic effects in breast cancer. Members of the retinoid receptor subfamily have also received considerable attention, particularly the crosstalk between the retinoic acid receptor alpha (RARA) and estrogen signaling (Hua *et al.* 2009, Ross-Innes *et al.* 2010), and the anti-migratory effect of the retinoic acid receptor beta (RARβ) in breast cancer (Yang *et al.* 2002, Flamini *et al.* 2014). However, the functional roles of the majority of the remaining members of the human NR family in the normal breast and breast cancer have largely been unexplored. Recent studies are establishing that the involvement of NRs in breast cancer extends beyond their involvement in regulating proliferation and apoptosis and that NRs are important regulators of several aspects of breast cancer tumorigenesis and progression including regulation of the circadian clock, metabolism, migration and metastasis. In this review, we will discuss evidence for these emerging functional roles of NRs in breast cancer, as well as new insights into the molecular mechanisms of action of NRs made possible through advances in genomic technologies.

The nuclear receptor superfamily

The human nuclear receptor superfamily consists of 48 members. These transcription factors include the receptors for steroid hormones, thyroid hormones, lipophilic vitamins and cholesterol metabolites including retinoic acid and oxysterols (Mangelsdorf *et al.* 1995). Members of the NR superfamily are identified through their highly evolutionarily conserved structural organization (Evans & Mangelsdorf 2014), which consists of four major domains: The N-terminal A/B domain with activation function1 (AF1); the DNA-binding domain (DBD) consisting of

two zinc finger motifs which confers response element specificity and the hinge region linking the DBD to the ligand-binding domain (LBD), which may or may not have an AF2 region that mediates coactivator interaction (Giguere *et al.* 1986, Kumar *et al.* 1987, Mangelsdorf *et al.* 1995) (Fig. 1A). Approximately half of the NRs are designated as orphans because endogenous physiological ligands for these NRs have not been found (Table 1). The 48 human NRs are organized into six evolutionary groups based on sequence alignment and phylogenetic tree construction (Auwerx *et al.* 1999, Germain *et al.* 2006). Organization of NRs into functional groupings is also feasible, based on profiling the anatomical expression of NRs. In the mouse, this revealed a hierarchical organization of NRs into integrated physiologic functional groups (Fig. 1B), which partially reflect the functional groupings observed in human, especially for NRs that are involved in circadian and metabolic pathways (reviewed here).

In the classical model of NR action, upon ligand activation, NRs regulate gene transcription by binding response elements within the regulatory regions of target genes as monomers, homodimers or heterodimers with another family member. For example, steroid hormone receptors generally bind as homodimers, whereas VDR, THR, RAR and RXR can form both homodimers and heterodimers. RXRs in particular can act as promiscuous heterodimerization partners for VDR, THR, RAR and orphan receptors. Dimerization is a general mechanism to increase binding affinity, specificity and diversity. Nuclear receptor response elements are derivatives of the canonical hexameric sequence RGGTCA. Modifications and duplications (organized as direct, inverted or everted repeats separated by a spacer with variable length) of this canonical hormone response element allow for selective recognition by different NR subclasses (Laudet & Gronemeyer 2002, Gronemeyer *et al.* 2004).

Ligand binding also allosterically controls the interaction of NRs with coregulators by influencing the conformation of the AF2 region. Coregulators are integral to the mechanisms by which NRs exert their functions and include both coactivators and corepressors. The majority of coregulators function as members of large complexes that affect NR transcriptional regulation of target genes through interactions with other transcription factors, the chromatin landscape as well as non-coding RNAs (Millard *et al.* 2013). Both the interaction with coregulators and the chromatin landscape of the cell have been shown to affect the cell type specificity of NRs.

NRs regulate the transcription of genes that control a wide variety of biological processes in normal physiology

Figure 1

(A) Schematic diagram of the structural and functional organization of nuclear receptors (reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Drug Discovery* (Gronemeyer *et al.* 2004), copyright 2004). The evolutionary conserved regions C and E are indicated as boxes (green and orange, respectively), and a black bar represent the divergent regions A/B, D and F. Domain functions are depicted above and below the scheme (AD, activation domain; AF1, activation function 1; NLS, nuclear localization signal). (B) Hierarchical clustering of NR tissue expression profile (reprinted from *Cell*, volume 126, Bookout AL, Jeong Y, Downes M, Yu RT, Evans RM & Mangelsdorf DJ, Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network, pages 789–799, copyright (2006), with permission from Elsevier). The relationship between receptor expression, function and physiology is depicted as a circular dendrogram using the hierarchical, unsupervised clustering of NR tissue expression distribution profiles in mouse. The analysis reveals the existence of a higher order network tying nuclear receptor function to reproduction, development, central and basal metabolic functions, dietary-lipid metabolism and energy homeostasis. The NRs in red boxes are discussed in this review with a focus on their functional roles in circadian regulation, metabolism and breast cancer migration and metastasis.

including embryonic development, reproduction, metabolism, homeostasis and cell proliferation. NRs are also known to play a role in various pathological processes including cancer, inflammation and metabolic disorders (McKenna & O'Malley 2010) and as such have been targets of intensive drug development for decades (Ottow & Weinmann 2008).

NR expression in the normal breast and breast cancer

Recent studies have begun to provide a global view of the functional roles and expression of the NR superfamily in primary normal breast (Muscat *et al.* 2013) and breast cancer tissues (Muscat *et al.* 2013, Lin *et al.* 2015). Muscat and coworkers found that the majority of NRs are expressed in the breast (41 of 48). Many NRs showed differential expression in breast cancer compared to the normal breast indicative of potential involvement in breast cancer biology (Muscat *et al.* 2013). In particular, breast cancer is associated with the overexpression of the NR4A subgroup as well as *EAR2*, and pan-repression of the majority of NRs relative to the normal breast. High expression of the NR4A subgroup in breast cancer was

also observed by Lin and coworkers (Lin *et al.* 2015). In addition to *ESR1*, *PGR* and *AR*, expression levels of other NRs were shown to be associated with histological grade (*THRB*, *NUR77*, *RORC*, *COUP-TF2* and *LRH-1*), to classify breast tissues (*THRA*, *ESR1*, *NURR77*, *EAR2* and *RARG*) and to predict metastasis-free survival in tamoxifen-treated patients (*THRB*, *COUP-TF2*, *MR* and *PPARG*). Thirty-three of the 48 human NRs were also observed to be expressed in stromal cells and four of these (*RORA*, *THRB*, *VDR* and *PPARG*) were shown to have differential expression profiles in cancer-associated fibroblasts compared to normal breast adipose fibroblasts (Knower *et al.* 2013). Together, these studies highlight that multiple members of the NR family are likely to play important roles in breast cancer growth and development and to have discriminant and prognostic value in breast cancer.

NR expression in breast cancer subtypes

Breast cancer is a heterogeneous disease encompassing multiple subtypes with distinct molecular profiles, therapeutic response and clinical outcomes (Prat *et al.* 2015). Several NRs have been shown to either act co-operatively with *ESR1* and/or have differential

Table 1 Human nuclear receptors.

Name	Nomenclature	Symbol	Entrez ID	Ligands	Expression in BC vs normal	Association with <i>ESR1</i> expression
Steroid hormone receptors						
Estrogen receptor	NR3A1	ESR1	2099	Estrogens	Increased	Yes ^{a,b}
	NR3A2	ESR2	2100	Estradiols	Decreased	
3-Ketosteroid receptor	NR3C1	GR	2908	Cortisols (hydrocortisone)	Increased	
	NR3C2	MR	4306	Aldosterone	Decreased	
	NR3C3	PGR	5241	Progesterone	Expressed in BC	Yes ^{a,b}
	NR3C4	AR	367	Testosterone, dihydrotestosterone	Increased	Yes ^{a,b}
Non-steroid hormone receptors						
Thyroid hormone receptor	NR1A1	THRA	7067	Thyroxine (T ₄), triiodothyronine (T ₃)	Decreased	
	NR1A2	THRB	7068	Thyroxine (T ₄), triiodothyronine (T ₃)	Decreased	Yes ^{a,b}
Retinoic acid receptor	NR1B1	RARA	5914	All-trans and 9-cis retinoic acid	Increased	Yes ^{a,b}
	NR1B2	RARB	5915	All-trans and 9-cis retinoic acid	Decreased	
	NR1B3	RARG	5916	All-trans and 9-cis retinoic acid	Decreased	
RAR-related orphan receptor	NR1F1	RORA	6095	Oxysterols	Decreased	
	NR1F2	RORB	6096	Cholesterol, cholesteryl sulphate	Decreased	Yes ^b
	NR1F3	RORC	6097	Retinoic acid	Decreased	Yes ^b
Vitamin D receptor	NR1I1	VDR	7421	Calcitriol (1',25' dihydroxy vitamin D3)	Decreased	
Adopted orphans						
Peroxisome proliferator- activated receptor	NR1C1	PPARA	5465	Fatty acids	Decreased	Yes ^b
	NR1C2	PPARD	5467	Fatty acids	Decreased	
	NR1C3	PPARG	5468	Fatty acids	Decreased	
Rev-erbs	NR1D1	REV-ERBa	9572	Heme	Decreased	
	NR1D2	REV-ERBb	9975	Heme	Decreased	
Liver X receptor-like	NR1H2	LXRb	7376	Retinoic acid	Decreased	
	NR1H3	LXRa	10062	Oxysterols	Decreased	
	NR1H4	FXRa	9971	Bile acids	Not expressed	
Vitamin D receptor-like	NR1I2	PXR	8856	Bile acids	Decreased	
	NR1I3	CAR	9970	Androstanol, androsthenol	Not expressed	
Retinoid X receptor	NR2B1	RXRA	6256	9-cis-retinoic acid	Decreased	
	NR2B2	RXRB	6257	9-cis-retinoic acid	Decreased	
	NR2B3	RXRG	6258	9-cis-retinoic acid	Decreased	
Steroidogenic factor-like	NR5A1	SF-1	2516	Phospholipids	Not expressed	
	NR5A2	LRH-1	2494	Phospholipids	Decreased	Yes
Orphan nuclear receptors						
DSS-AHC critical region on the X chromosome, gene 1	NR0B1	DAX-1		Not known	Not expressed	Yes
Short heterodimeric partner	NR0B2	SHP	8431	CD437 retinoids	Not expressed	
Hepatocyte nuclear factor 4	NR2A1	HNF4a	3172	Fatty acids	Not expressed	
	NR2A2	HNF4g	3174	Fatty acids	Decreased	
Testicular orphan receptors	NR2C1	TR2	7181	All-trans retinoic acid	Decreased	
	NR2C2	TR4	7182	All-trans retinoic acid	Decreased	
Tailless-related receptor	NR2E1	TLX	7101	Not known	Not expressed	Yes ^b
Photoreceptor cell-specific receptor	NR2E3	PNR	10002	Benzimidazoles	Decreased	Yes ^{a,b}
Chicken ovalbumin upstream promoter-transcription factor	NR2F1	COUP-TF1	7025	Not known	Decreased	
	NR2F2	COUP-TF2	7026	Retinol/ATRA	Decreased	

(Continued)

Table 1 Continued.

Name	Nomenclature	Symbol	Entrez ID	Ligands	Expression in BC vs normal	Association with ESR1 expression
ErbA2-related gene 2	NR2F6	EAR2	2063	Not known	Increased	
Estrogen receptor-related receptor	NR3B1	ESRRA	2101	Isoflavones, diethylstilbestrol, chlordane	Decreased	
	NR3B2	ESRRB	2103	Isoflavones, diethylstilbestrol, 4-hydroxy tamoxifen	Decreased	
	NR3B3	ESRRG	2104	Isoflavones, diethylstilbestrol, 4-hydroxy tamoxifen	Decreased	Yes ^b
Nerve growth factor IB-like	NR4A1	NUR77	3164	Cyclosporone-B, diindolylmethane analogs	Increased	
	NR4A2	NURR1	4929	6-Mercaptopurine, benzimidazoles, C-DMIs	Increased	
	NR4A3	NOR1	8013	6-Mercaptopurine, prostaglandin A2	Increased	
Germ cell nuclear factor	NR6A1	GCNF	2649	Not known	Decreased	

^aMuscat *et al.* (2013); ^bLin *et al.* (2015).

actions in ER+ vs ER- breast cancers. For instance, AR has different roles in ER+ vs ER- breast cancers. AR has been investigated extensively as a therapeutic target in ER- breast cancer. It was reported that in ER-/HER2+ breast tumors, AR stimulates oncogenic Wnt and HER2 signaling pathways thereby stimulating cancer cell growth (Ni *et al.* 2011). However, the role of androgen signaling in ER+ breast cancer is less clear. Expression of AR is associated with good prognosis in ER+ breast cancer (Hu *et al.* 2011, Hilborn *et al.* 2016), with anti-estrogenic and anti-proliferative effects in ER+ breast cancer being reported (Peters *et al.* 2009, D'Amato *et al.* 2015). The anti-proliferative effect of AR is partly attributed to its inhibitory effect on ESR1 signaling in ER+ breast cancer cells (Peters *et al.* 2009, Fioretti *et al.* 2014). However, AR has also been reported to contribute to therapy resistance in ER+ breast cancer cells overexpressing AR, with increase in AR-to-ESR1 ratio associated with worse outcome for tamoxifen-treated patients (Cochrane *et al.* 2014) and increased agonist activity of Tam in AR overexpressing cells. In this context, AR reportedly increased tamoxifen agonist activity via the activation of EGFR in ER+ breast cancer, and this was blocked by dual treatment with the anti-androgen enzalutamide and EGFR inhibitor gefitinib (Ciupek *et al.* 2015). In addition, AR appears to promote proliferation of molecular apocrine breast cancer; a subset of TNBC expressing AR, through activation of genes that are normally regulated by ESR1 in ER+ cancer (Robinson *et al.* 2011).

Similarly, the expression of GR has been shown to be differentially associated with survival in ER+ vs ER- breast cancers. In ER+ breast cancers, high GR expression is associated with better survival (Pan *et al.* 2011, Abduljabbar *et al.* 2015b), whereas in ER- breast cancers, high GR expression is correlated with poorer breast cancer-specific survival (Pan *et al.* 2011, Abduljabbar *et al.* 2015b).

Recent studies profiling the expression of NRs in ER+ and ER- cancers have also identified other NRs whose expression levels differ significantly between ER+ and ER- breast cancers (Table 1). In particular, AR, PGR, PNR, RARA and THRB were reported to be significantly differentially expressed between ER+ and ER- breast cancers (Muscat *et al.* 2013) and significantly associated with ESR1 expression (Lin *et al.* 2015). The expression of DAX-1, LRH-1, PPARA, RORB, RORC and TLX were also reported to be significantly associated with ESR1 expression in multiple microarray datasets (Lin *et al.* 2015).

Mechanisms of NR action

Alterations in normal transcriptional programs are a fundamental feature of cancer pathogenesis and progression. Nuclear receptors are important regulators that directly couple small-molecule signaling with transcriptional regulation. An understanding of their mechanisms of action at the genomic and network level, and how these processes are altered in breast cancer, is of

fundamental importance to our understanding of breast cancer development.

The development of technologies such as ChIP-chip, ChIP-Seq and GRO-Seq has enabled profiling of the genomic locations of NR binding in an unbiased, genome-wide manner. Studies employing these methods have revealed new insights into the mechanisms of action of nuclear receptors and highlight the complexity of nuclear receptor regulatory networks in breast cancer. In this subsection, we review new insights of NR mechanisms of action in breast cancer made possible by these genomic methods.

Shifts from the 'classical' model of NR action

Traditionally, studies on nuclear receptor action at hormone-responsive genes gave rise to the model that NRs bind proximally to the promoters of target genes, through recognition of stringent paired half-sites with highly specific orientation (Mangelsdorf *et al.* 1995). However, these classical models have been challenged by recent studies that profiled the genome-wide binding sites of several nuclear receptors and transcription factors using ChIP-chip or ChIP-Seq. In the context of breast cancer, genome-wide mapping of ESR1 genomic localization in the MCF-7 breast cancer cell line showed that most ESR1 binding occurs at distal regions of the genome and that many ESR1 sites did not contain the canonical ESR1-binding motif (Carroll *et al.* 2006). Kittler and coworkers examined the genomic distribution of the binding sites of 24 NRs in MCF-7 cells and found that NR varied significantly in their genomic localization, with both proximal-biased and distal-biased NRs being observed (Kittler *et al.* 2013). The observation that NRs localize not only to proximal promoter regions but also to distal regions and the prevalence of non-canonical motif and half-site recognition is also confirmed in a meta-analysis of NR cistromes in different cell types (Tang *et al.* 2011). NR binding at non-canonical response elements can be a mechanism to fine tune transcriptional responses of NR. For example, the glucocorticoid receptor (GR) is capable of binding a negative response element (nGRE) that confers transrepression in addition to the canonical glucocorticoid response elements (GRE) that confers transactivation to liganded GR in epidermis and intestinal epithelial cells (Surjit *et al.* 2011). Furthermore, NRs are capable of mediating long-range transcriptional regulation that involves chromatin looping as illustrated by studies on the estrogen receptor-mediated long-range chromatin interactions (Liu & Cheung 2014). Together, these studies suggest a more complex model of NR genomic action in which NRs frequently act on long-range

enhancers whose actions can be fine-tuned by recognition of different response elements.

Cell type specificity and the chromatin landscape

Studies investigating NR cistromes highlight the remarkable difference in the genome-wide binding profiles of the same NR in different cell types (Krum *et al.* 2008, Clarke & Graham 2012). In addition to the influence of coregulators in determining the cell type-specific transcriptional response of NRs (discussed in the next section), another major aspect of NR action illuminated through advances in genomic technologies is the dynamic and complex interactions between NR and the chromatin landscape of the cell. It is becoming clear that chromatin, existing in a dynamic continuum of condensation states, regulates as well as is regulated by, NR actions, and that NR–chromatin interaction is a major determinant of NR cell type-specific transcriptional regulation. Due to the widely accepted model of NR binding to DNA only after conformational changes induced upon ligand binding, several groups have investigated the question of pre-induction vs post-induction chromatin as a determinant of NR action. Genome-wide screening of DNase I hypersensitive sites (DHS) in breast cells revealed that the majority (>70%) of GR binding occurs at pre-induction DHS sites, whereas only ~20% of GR binding occurs at hormone-induced remodeled chromatin (John *et al.* 2011). A recent study of long-range interactions before and after dexamethasone induction in HeLa cells found that, similar to breast cells, the majority of GR binding occurs at pre-accessible chromatin. Binding of GR to a subset of sites containing the consensus GRE results in the formation of active enhancers and increased long-range interactions (Kuznetsova *et al.* 2015). Profiling of ESR1 revealed a similar pattern. The majority of ESR1-binding sites coincide with pre-existing open chromatin, co-occurrence of other transcription factor binding, and correspond to cell type-specific gene regulation, whereas ESR1-binding sites shared by multiple cell types are characterized by inaccessible chromatin containing high-affinity estrogen response elements (Gertz *et al.* 2013). Profiling of the PGR by DNase and Micrococcal nuclease (MNase) followed by next-generation sequencing showed that PGR binding upon hormone stimulation corresponds with DHS regions that still exhibit high nucleosome occupancy. Upon hormone treatment, remodeling of nucleosomes containing functional PGR-binding sites occurs, and progesterone-responsive genes are associated with PGR-binding sites that show strong nucleosome

remodeling upon hormone induction (Ballare *et al.* 2013). The chromatin landscape of the cell therefore plays an important role in determining the genomic binding profiles of specific NRs. Although some NRs such as ESR1 and GR rely mostly on pre-programmed sites made accessible by other cell- and tissue-specific pioneer DNA-binding factors, other NRs such as PGR require ligand-dependent active chromatin remodeling.

Pioneer factors and coregulators as modulators of NR function

Pioneer factors are transcription factors that can bind condensed chromatin and influence transcription by facilitating subsequent recruitment of other transcriptional regulators. Recent studies have highlighted the importance of pioneer factors in facilitating chromatin accessibility and also in determining the cell type-specific response of NRs. This is exemplified by the dependence of ESR1 on FOXA1 in breast cancer. FOXA1 expression is luminal restricted (Perou *et al.* 2000) and is thought to function as a pioneer factor (Cirillo *et al.* 2002). FOXA1 has been observed to influence genome-wide chromatin accessibility and is a key determinant of cell type-specific ESR1 genomic localization (Lupien *et al.* 2008, Hurtado *et al.* 2011), reflecting the importance of interaction with other factors on control of cell specificity of ESR1. Similarly, AP-1 has been shown to recruit GR to regions of the genome that are basally accessible prior to hormonal treatment. AP-1 is thought to maintain chromatin accessibility for GR binding as well as recruiting GR indirectly in the absence of a canonical GR motif (Biddie *et al.* 2011).

In addition to pioneer factors, coregulators interact with NRs and other transcription factors to facilitate transcription of target genes. According to the Nuclear Receptor Signaling Atlas (NURSA: www.nursa.org), there are 320 NR coregulators identified to date. However, this number is likely to be a gross underestimation according to a recent proteomic study (Malovannaya *et al.* 2011). Coregulators can assist in transcriptional regulation of NRs by facilitating chromatin accessibility, stabilizing NR–DNA interaction or facilitating indirect NR–DNA interaction through tethering (McKenna & O'Malley 2002, Millard *et al.* 2013).

Selective recruitment of coregulators to subsets of NR-binding sites can also be a mechanism through which transcriptional regulation of subsets of NR target genes are fine-tuned. An early study of ESR1 and steroid receptor coactivator protein (SRC) binding using promoter tiling arrays showed E2-dependent recruitment of ESR1 and

SRC to E2-stimulated genes while the absence of SRC was observed at E2-repressed genes (Kininis *et al.* 2007). In a subsequent study, Zwart and coworkers mapped the genome-wide binding sites of several ESR1 coregulators (SRC1, SRC2, SRC3, p300 and CBP) in MCF-7 cells and showed a complex network of ESR1-coregulator binding, with preferential binding sites for each coregulator. They identified a subset of ESR1-regulated genes that are co-occupied by SRC3, but not SRC1 or SRC2, that predicts poor or good survival outcome depending on whether the genes were upregulated or downregulated (Zwart *et al.* 2011). Lupien and coworkers also identified association between a subset of E2-induced ESR1 binding sites co-occupied by CARM1 and gene repression, independent of the presence of FOXA1 binding. They also identified another subset of E2-induced, FOXA1-independent, ESR1 and CARM1 co-occupied sites that result primarily in repression of expression (Lupien *et al.* 2009). These studies demonstrate that selective interaction with different coregulators at specific subsets of target genes may be an important mechanism through which the specificity of NR action is modulated.

Due to their central role in regulating NR-mediated transcription, many coregulators are known to be involved in human diseases (Lonard *et al.* 2007, Lonard & O'Malley 2012). In the breast, changes in expression levels of coregulators have been implicated in the tissue-specific response to tamoxifen (Keeton & Brown 2003). The majority of coregulators show differential expression between breast cancer and normal breast tissues, as well as between ER+ and ER– breast cancers. This is accompanied by changes in the expression correlations between subsets of coregulators and specific NRs, whose expression showed prognostic value in breast cancer (Doan *et al.* 2014).

Coregulators are therefore critical determinants of NR-mediated transcriptional regulation in the breast and disruptions in the normal NR–coregulator interaction network are potentially an important aspect of breast cancer biology.

Combinatorial control of gene expression by NRs in breast cancer

Recent large-scale integrative analyses of transcription factor genomic localization data from the ENCODE project highlighted the extraordinary complexity of transcriptional regulation and the extensive functional crosstalk between transcription factors (ENCODE Project Consortium 2012, Gerstein *et al.* 2012, Xie *et al.* 2013). This observation also holds for NR in breast cancer.

Kittler and coworkers (Kittler *et al.* 2013) built a regulatory map from cistromic data of 24 NRs and 14 breast cancer-associated coregulators in MCF-7 breast cancer cells. They showed that the resulting network is highly interconnected and there are many regions in the genome coordinately occupied by multiple NRs (HOT regions). These HOT regions are enriched with features associated with active regulatory elements, including active chromatin marks and increased chromatin accessibility. These regions are also enriched with breast cancer-relevant genes and are hypothesized to be important active regulatory regions in breast cancer cells.

On a smaller scale, other studies have also revealed convergence in genomic binding between ESR1 and various other NRs in breast cancer cells by individually comparing the overlap of their cistromes. ESR1 is known to drive growth and proliferation in the majority of breast tumors. Mounting evidence indicates that ESR1 does not act on its own and that other transcription factors, including other NRs, are important determinants of ESR1 action in breast cancer cells. For example, retinoic acid has been known to inhibit proliferation in breast cancer cells and antagonize the growth stimulation effect of estrogen (Fontana *et al.* 1992). Studies of ESR1 and RARA cistromes showed that ESR1 and RARA share very similar binding profiles, although whether the interaction is antagonistic or co-operative is still controversial (Hua *et al.* 2009, Ross-Innes *et al.* 2010). Likewise, cistromic profiling of ESR1 and GR in mouse mammary epithelial cell lines revealed significant co-operation of these two NRs, through an assisted loading mechanism, in which binding of one NR facilitates chromatin remodeling thereby enabling access to DNA for the other NR (Miranda *et al.* 2013). Another study profiling ESR1 and GR genomic localization in MCF-7 cells revealed that, although GR co-occupies several ESR1-binding sites in cells treated with both E2 and dexamethasone, GR recruitment to these sites is associated with displacement of ESR1 leading to the repression of estrogen receptor-mediated transcriptional activation of target genes (Karmakar *et al.* 2013). Therefore, although ESR1 co-occupies many target genes with RARA as well as GR, the nature of their interaction is reported to be both co-operative and antagonistic, perhaps reflecting different time, target and cell-specific modes of co-operation between these NRs.

In addition to RARA and GR, ESR1 was reported to share genomic binding sites with PGR and LRH-1. In the presence of both estrogen and progesterone, PGR was shown to be recruited to the ESR1 complex and to redirect ESR1-binding events, resulting in a gene expression profile

associated with better clinical outcome (Mohammed *et al.* 2015). However, in the presence of estrogen alone, un-liganded PGRB activated a subset of ER target genes by acting as a molecular scaffold for the formation of a transcriptional complex with ESR1 and PELP1, resulting in a more aggressive proliferative response to estrogen (Daniel *et al.* 2015). It is likely that the action of ESR1 and PGR and their crosstalk are highly context dependent. Furthermore, the majority of data are derived from cell line models, and the implications for breast cancer *in vivo* are yet to be established. LRH-1 was reported to share a substantial portion (~35%) of its binding sites with that of ESR1 and synergistically regulate a subset of estrogen-responsive genes in MCF-7 cells (Lai *et al.* 2013).

These studies highlight the complexity of cross talk, particularly between ER and other NRs, in gene regulatory networks and the utility of integrated, genome-wide analyses in unraveling this complexity. Whether the studies so far reflect transcriptional plasticity that is particularly a feature of ER or whether data will emerge supporting this as a mechanism common to other NRs is still to be determined; nevertheless, these insights into the functional interactions between NRs in breast cancer have the potential to lead to novel therapeutic strategies.

Reprogramming of NR binding and disease progression

It is becoming increasingly evident that transcriptional regulation is a highly dynamic process with transcription factors displaying temporal, cell type specific and disease-associated shifts in their genomic binding profiles. The transcriptional targets of PGR in T-47D breast cancer cells and AB-32 immortalized normal breast cells display remarkably low overlap (Clarke & Graham 2012), reflecting cancer-associated and cell type-specific changes in PGR transcriptional regulation. Recent studies have highlighted the effect of changes in NR transcriptional programs and their association with breast cancer disease progression and clinical outcomes. Ross-Innes and coworkers performed ChIP-seq of ESR1 in clinical samples with different prognoses to directly explore the correlation between ESR1 binding and cancer progression (Ross-Innes *et al.* 2012). They observed high signal intensity at ESR1-binding sites in metastatic samples, whereas lower ESR1 signal intensity was observed in samples with good prognosis. Further, they showed that ESR1 binding is a highly dynamic process, with distinct ESR1-binding regions observed in samples with different outcomes. This dynamic change in ESR1 action was shown to be influenced by

the action of other NRs, such as PGR (Mohammed *et al.* 2015), as well as the signaling context leading to ESR1 activation. For example, ESR1 displayed distinct genomic binding profiles depending on whether ESR1 was activated by estrogen or through the epidermal growth factor (EGF) pathway (Lupien *et al.* 2010). The EGF-induced ESR1 cistrome specifically regulates genes that are overexpressed in ERBB2-positive breast cancers and associated with poor clinical outcomes. The EGF pathway can therefore be an alternative activator for ESR1 signaling in breast cancer and provides a molecular explanation for the endocrine therapy resistance often seen in ER+ERBB2+ breast cancers. Analysis of ESR1 and ESRRA cistromes in tamoxifen-sensitive vs -resistant breast cancer cells showed that despite regulating distinct transcriptional networks, their cistromes are reprogrammed in tamoxifen-resistant breast cancer cells toward the regulation of genes functionally relevant to resistance (Thewes *et al.* 2015). These studies show that NR binding profiles can be shifted in a context-dependent manner, resulting in altered transcriptional networks that affect disease progression and outcome.

Emerging functional roles of NR in BC

It is now clear that a number of NRs are implicated in breast cancer growth and development (Conzen 2008). It is emerging that, in addition to influencing cell growth and proliferation, NRs also play important roles in other aspects of breast cancer biology. Table 2 summarizes reported involvement of NRs in selected aspects of breast cancer biology. This section outlines some of the emerging functional roles of NRs in breast cancer.

Nuclear receptors as key components of the circadian clock

A circadian rhythm is any biological process that displays an endogenous, entrainable oscillation cycle of roughly 24 h, driven by a self-sustained timekeeping mechanism, the circadian clock. The circadian clock governs many aspects of human physiology, including body temperature, sleep–wake cycle, blood pressure, hormone secretion and metabolism. In line with this, at least 10% of all genes are under the control of the circadian system and display

Table 2 NR function in breast cancer.^a

Nomenclature	Symbol	Anti-proliferative	Pro-apoptotic	Anti-migratory	Pro-oxidative phosphorylation	Pro-proliferative	Anti-apoptotic	Pro-migratory	Pro-glycolytic	Circadian involvement
NR3A1	ESR1					Y		Y		Y
NR3A2	ESR2	Y		Y				Y		
NR3C1	GR						Y			Y
NR3C3	PGR			Y		Y	Y	Y		
NR3C4	AR	Y				Y				
NR1B1	RARA	Y	Y							
NR1B2	RARB	Y	Y	Y						
NR1B3	RARG	Y	Y							
NR1F1	RORA									Y
NR1F2	RORB									Y
NR1F3	RORC									Y
NR1I1	VDR	Y	Y							
NR1C1	PPARA					Y				
NR1C2	PPARD					Y				
NR1C3	PPARG	Y	Y			Y	Y			
NR1D1	REV-ERBa	Y			Y					Y
NR1D2	REV-ERBb	Y							Y	Y
NR2B1	RXRA		Y							
NR2B2	RXRB		Y							
NR2B3	RXRG		Y							
NR2F1	COUP-TF1							Y		
NR2F2	COUP-TF2							Y		
NR3B1	ESRRA							Y	Y	
NR3B3	ESRRG			Y	Y					
NR4A1	NUR77							Y		

^aNR effect on cancer-related biological processes based on studies in breast primary tissue or cell lines. The influence of NR on breast cancer proliferation, apoptosis, migration, metabolism as well as involvement in circadian regulation/response is tabulated.

Y, indicates reported involvement.

circadian oscillation (Akhtar *et al.* 2002, Oishi *et al.* 2003, 2005). There is mounting evidence linking circadian disruption to various clinical and pathological conditions including cancer. Here, we discuss the emerging link between NRs and circadian regulation, as well as the growing body of evidence linking circadian disruption and breast cancer.

Initially, the circadian clock was defined to be a relatively simple feedback loop consisting of the key transcription factors CLOCK and BMAL1 and their co-repressors, PER and CRY (Fig. 2). It is now emerging that the circadian clock contains other auxiliary loops that involve multiple NRs, most prominently REV-ERBs and RORs (Fig. 2, reused from Bechtold *et al.* (2010), with permission from Elsevier). RORs recognize the same

regulatory elements as REV-ERBs, and a range of studies have demonstrated their importance in the regulation of clock gene expression (Akashi & Takumi 2005). Gene deletion studies and cistrome analyses have confirmed the pivotal role of REV-ERBs in the universal circadian machinery (Preitner *et al.* 2002).

In addition to REV-ERBs and RORs, several other NRs have been shown to regulate as well as being regulated by the circadian clock, most notably GR (Conway-Campbell *et al.* 2010) and ESRRA (Dufour *et al.* 2011). The list of other NRs potentially under control of the circadian clock is extensive, with at least 35 NRs known to display circadian expression in various metabolic tissues (Zhao *et al.* 2014).

Figure 2

The molecular clock machinery (reprinted from *Trends in Pharmacological Sciences*, volume 31, Bechtold DA, Gibbs JE & Loudon AS, Circadian dysfunction in disease, pages 191–198, copyright (2010), with permission from Elsevier). The molecular machinery that provides circadian timekeeping consists of a complex circuitry of transcriptional/translational regulatory feedback loops (clock components shown in gray). In mammals, the current model involves a primary loop with CLOCK (or homologue NPAS2) and BMAL1 as transcriptional activators, and PERIOD (PER1, PER2 and PER3) and CRYPTOCHROME proteins (CRY1 and CRY2) as transcriptional repressors. As levels of cytosolic PER and CRY proteins rise, they associate, translocate to the nucleus and repress their own gene transcription through direct interaction with the CLOCK/BMAL1 complex. This feedback cycle provides near 24-h timing and drives the rhythmic expression of several clock-controlled and clock-modulated genes, which in turn mediate circadian rhythms in behavior and physiology. Acting on the primary feedback loop are auxiliary loops, which appear to increase the stability and robustness of the oscillations. The most notable interlocking loop is that involving the nuclear hormone receptors (NRs), REV-ERB and ROR. In addition to REV-ERB and ROR, several other NRs (shown in green) interact closely with the circadian feedback loops and are responsive to the clock (exhibit rhythmic expression) and able to feedback onto the clock genes themselves. NR regulation of clock genes also renders the clock responsive to numerous circulating hormones (e.g. cortisol, estrogen), nutrient signals (e.g. derivatives of fatty acids and retinoids) and cellular redox status (NADH/NAD⁺ ratio).

To date, epidemiological studies reported mixed findings on the association between increased risk of breast cancer and circadian disruption in the form of night shift work. Although a few studies reported no significant association between breast cancer and night work (O'Leary *et al.* 2006, Pronk *et al.* 2010), the majority of recent studies reported significant or borderline associations, with the strongest association found with long-term exposure to night work (Hansen 2001, Schernhammer *et al.* 2001, 2006, Hansen & Lassen 2012, Hansen & Stevens 2012, Knutsson *et al.* 2013, Menegaux *et al.* 2013, Rabstein *et al.* 2013, Akerstedt *et al.* 2015, Papantoniou *et al.* 2015). Meta-analysis studies have also reported a positive association between night work and breast cancer risk (Jia *et al.* 2013) and that a positive dose–response gradient is observed for breast cancer with increased years of night shift and cumulative night work (Wang *et al.* 2013).

Although circadian disruption is prominently linked to breast cancer tumorigenesis through epidemiological studies, the molecular mechanisms and NRs involved are still largely unknown, with most published studies focusing on the ESR1 as a link between the circadian system and breast cancer development. Many studies suggest that lowered melatonin level due to exposure to night light contributes toward breast cancer development and drug resistance. Melatonin is thought to exert its oncostatic effect through multiple mechanisms including anti-mitosis, anti-angiogenesis, anti-oxidation and regulation of immune response (Viswanathan & Schernhammer 2009). The oncostatic effect of melatonin in breast cancer is thought to be mediated through its anti-estrogenic actions as well as its role in regulating the activity of aromatases, the enzymes responsible for the local biosynthesis of estrogens from androgens (Cos *et al.* 2006). Recent studies using MCF-7 breast cancer xenografts in mice showed that suppression of melatonin production by dim light exposure at night leads to both tamoxifen (Dauchy *et al.* 2014) and doxorubicin resistance (Xiang *et al.* 2015).

In addition to the effect of melatonin, there is evidence linking the core clock genes as well as the REV-ERBs with breast cancer growth and metastasis. The rhythmic expression of the PER genes are reported to be altered in primary breast tumors (Chen *et al.* 2005) as well as in breast cancer cell lines (Xiang *et al.* 2012). A recent study demonstrated the association of the core clock gene BMAL2 in ER breast cancer metastasis (Ha *et al.* 2016). Furthermore, activation of REV-ERBA and REV-ERBB by a synthetic ligand is anti-proliferative in breast cancer cells.

ESR1 has been shown to be an important regulator linking the circadian system and breast cancer tumorigenesis, acting as a regulator of (as well as being regulated by) the core clock genes. ESR1 appears to participate in a feedback loop involving the clock protein PER2, which was shown to be a tumor suppressor in luminal breast cancer. Although PER2 is E2 inducible, PER2 itself is thought to mediate ESR1 degradation through the proteasome pathway. Suppression of *PER2* leads to ESR1 stabilization (Gery *et al.* 2007). In addition, ESR1 was shown to bind estrogen response elements in the promoter region of *CLOCK* leading to the upregulation of *CLOCK* in breast cancer cells. Knockdown of *CLOCK* attenuated proliferation in breast cancer cells (Xiao *et al.* 2014). Therefore, *CLOCK* appears to be an important mediator of the proliferative effect of E2 in breast cancer.

In summary, NRs play important regulatory roles in both the universal and tissue-specific circadian systems. In particular, REV-ERBs and RORs are integral regulators of the universal circadian clock, and various other NRs exhibit circadian expression. Although epidemiological studies have linked circadian disruption and breast cancer tumorigenesis, a lot remains to be learned about the underlying molecular mechanism. Lowered melatonin due to night light exposure is thought to play a role and there is evidence supporting the involvement of ESR1 as an integral regulator linking circadian regulation and breast cancer development.

Regulation of energy metabolism

As the initial observation by Warburg that proliferating tumor cells converted the majority of their glucose to lactate even in oxygen-rich condition and his hypothesis that altered metabolism is a characteristic of cancer cells, metabolic reprogramming has now attained cancer hallmark status (Ward & Thompson 2012). Specifically, the Warburg effect describes a shift toward aerobic glycolysis in preference to oxidative phosphorylation as the major means of ATP generation in cancer cells, even in oxygen-rich conditions. Although aerobic glycolysis is not as efficient as oxidative phosphorylation (yielding only 2 mol of ATP/mole of glucose instead of 36 mol ATP/mole of glucose), it has certain benefits for tumor cells. Glycolysis results in more rapid production of ATP, fatty acids and nucleotides that are needed by proliferating tumor cells that need to double their biomass to divide. Furthermore, the generation of lactic acid promotes a tumor microenvironment that is protective against

immune attack (Calcinotto *et al.* 2012). It has also been suggested through glucose flux modeling that accumulation of glucose actually promotes aerobic glycolysis in preference to oxidative phosphorylation (Vazquez *et al.* 2010); hence, glucose flux can potentially act as a trigger for the Warburg effect.

It has emerged that NRs are centrally placed in many of the pathways controlling energy metabolism, from regulating glucose transporters to controlling the downstream pathways involved in glucose metabolism. Their altered expression in breast cancers is potentially intimately linked to the metabolic reprogramming of the tumors cells. Here, we review our current understanding of the roles of NRs in breast cancer cellular metabolism.

REV-ERBs In addition and related to their prominent roles in circadian regulation, REV-ERBs are recognized as integrators of circadian regulation and metabolic pathways (Cho *et al.* 2012, Gerhart-Hines & Lazar 2015). A recent study looking at genome-wide location of REV-ERBA in various tissues reported different mechanisms of gene regulation employed by REV-ERBA with regard to circadian control and metabolic control. Although circadian regulation requires direct DNA binding of REV-ERBA to its cognate site, metabolic control involves the recruitment of HDAC3, which is tethered by cell type-specific transcription factors (Zhang *et al.* 2015). A recent publication suggests that although REV-ERBA has a dominant role in normal cells, REV-ERBB seems to be the dominant isoform controlling both circadian rhythm and metabolic pathways in cancer cells, including breast cancer (De Mei *et al.* 2015). REV-ERBB is significantly more abundant in cancer cells compared to REV-ERBA, whereas the opposite is observed in normal cells. Knockdown of REV-ERBB resulted in the enhanced expression of both circadian and metabolic target genes in breast cancer cells while knockdown of REV-ERBA had no effect. However, ERBB2-positive breast cancer cells appear to have higher expression of REV-ERBA compared to ERBB2-negative cells, consistent with a previous study reporting co-expression of REV-ERBA and ERBB2 and pro-survival function of REV-ERBA in ERBB2-positive breast cancer cells (Kourtidis *et al.* 2010). These studies suggest that the two REV-ERB isoforms probably have redundant functions in transcriptional regulation, and functional predominance in a particular cell type depends on the relative expression level of each isoform.

Estrogen-related receptors (ERRs) The estrogen-related receptors (ESRRA/NR3B1, ESRRB/NR3B2 and

ESRRG/NR3B3) have become recognized as the master regulators of energy metabolism in multiple tissues with high energy demand (Giguere 2008, Deblois & Giguere 2013). Both ESRRA and ESRRG have dominant roles in maintaining energy homeostasis through the regulation of metabolic gene networks. In the heart, ESRRA and ESRRG co-operate to control genes involved in uptake of energy substrates, production and transport of ATP across the mitochondrial membrane (Alaynick *et al.* 2007, Dufour *et al.* 2007). ESRRA was reported to co-operate with the peroxisome proliferator-activated receptor-gamma coactivator alpha gene (PGC-1 α) in a double-positive feedback loop to regulate the expression of many oxidative phosphorylation genes in mouse myoblast cells (Mootha *et al.* 2004). In the liver, ESRRA was reported to co-operate with PROX1 and BMAL1 to regulate circadian and metabolic gene networks (Dufour *et al.* 2011). Together, the three ERR isoforms recognize the proximal regulatory regions of 705 genes involved in all aspects of mitochondrial biogenesis and function (Eichner & Giguere 2011).

In the breast, ESRRA and ESRRG are associated with distinct survival outcomes (Ariazi *et al.* 2002). Although ESRRA is associated with increased recurrence and poor outcome (Thewes *et al.* 2015), ESRRG expression is correlated with ESR1 and ERBB4, which are markers of favorable outcome (Ariazi *et al.* 2002). It has been suggested that ESRRA and ESRRG have opposite roles in the regulation of metabolic reprogramming of breast cancer cells (Deblois & Giguere 2013). Although ESRRA promotes aerobic glycolysis through the upregulation of enzymes that regulate the glycolysis pathway (Cai *et al.* 2013), ESRRG is thought to sustain oxidative phosphorylation through regulation of genes that control the tricarboxylic acid cycle (Eichner *et al.* 2010). However, metabolic reprogramming by ERRs in breast cancers probably involves much greater complexity, given that overexpression of ESRRG also leads to increased expression of enzymes in the glycolytic pathways (Cai *et al.* 2013) and that ESRRA and ESRRG readily form heterodimers and bind to regulatory regions of many shared target genes (Dufour *et al.* 2007).

ESRRs also interact with oncogenes that are known modulators of metabolic pathways such as MYC, HIF and ERBB2. Many genes in the glycolytic pathways are regulated by MYC. ESRRA was found to participate in a complex with MYC to co-regulate glycolytic genes (Cai *et al.* 2013). HIF directly activates glucose transporters and the majority of genes in the glycolytic pathways. All three ESRR isoforms were identified as coactivating

factors of HIF and to enhance HIF-induced glycolytic and angiogenic gene expression in hypoxic condition (Ao *et al.* 2008). ESRRA also regulates the expression of *ERRB2* (Deblois *et al.* 2010), which was reported to translocate to the mitochondria of tumor cells and regulate energy metabolism. Overexpression of mitochondrial *ERRB2* decreases mitochondrial electron transport chain activity and enhances cellular glycolysis (Ding *et al.* 2012). In addition, *ERRB2* influences the glycolytic phenotype through upregulation of lactate dehydrogenase (Zhao *et al.* 2009) as well as *REV-ERBA*, an NR that was reported to be involved in glycolysis and fatty acid synthesis in breast cancer cells (Kourtidis *et al.* 2010).

In summary, the ERRs are prominent regulators of gene networks controlling glycolysis and oxidative phosphorylation in metabolic tissues. In the breast, *ESRRA* expression is associated with poor outcome and appears to be involved in reprogramming the cell to favor aerobic glycolysis, a metabolic state characteristic of cancer cells. *ESRRG*, on the other hand, is associated with better outcome and is involved in maintaining oxidative phosphorylation, a metabolic state characteristic of normal cells. However, given that *ESRRA* and *ESRRG* can form heterodimers and readily bind to many shared target genes, the complexity of the mechanisms for controlling their specificity of action is still to be deciphered.

Peroxisome proliferator-activated receptors (PPARs) PPARs function as heterodimers with the retinoid receptors (RXR) and regulate various genes that are involved in lipid metabolism and energy homeostasis. Through these pathways, PPARs influence various cancer-related cellular processes such as proliferation, differentiation and survival (Michalik *et al.* 2004). Three PPAR isotypes (PPARA/NR1C1, PPARD/NR1C2 and PPARG/NR1C3) are known, each with unique expression profile and functional roles. The PPARs showed decreased expression in breast cancer compared to normal breast (Muscat *et al.* 2013). The role of PPARD in breast cancer tumorigenesis is unclear. Although activation of PPARD has been shown to inhibit breast cancer cell line tumorigenicity (Yao *et al.* 2014), inhibition of PPARD by inverse agonists was reported to inhibit breast cancer cell invasion (Adhikary *et al.* 2013). PPARG is the best studied of the 3 PPAR subtypes and has been reported to have an anti-proliferative effect on breast cancer cells (Pon *et al.* 2015) and is associated with better outcomes based on survival analysis of PPARG protein expression level in BC tissue microarrays (Abduljabbar *et al.* 2015a). The downstream effect of PPARG seems to be dependent

on the cell compartment in which PPARG is activated though, with activation in cancer cells resulting in growth inhibition while activation in stromal cells results in growth enhancement of co-injected breast cancer cells (Avena *et al.* 2013). Recent studies have also suggested an involvement of PPARG and energy metabolism in breast cancer. Firstly, several genes controlling the glycolytic pathways are found to have peroxisome proliferator response element (PPRE) suggesting their regulation by PPARs (Sakharkar *et al.* 2013). Secondly, overexpression of PPARG in fibroblasts increased the production of L-lactate and mitochondrial dysfunction. In addition, PPARG induces the activation of HIF1A, a transcription factor that promotes glycolysis (Avena *et al.* 2013).

Control of breast cancer migration and metastasis

Breast cancer metastasis accounts for the majority of deaths from breast cancer. Early detection and a deeper understanding of the metastatic process are critical to develop therapeutic interventions. Metastasis involves a complex cascade of steps starting with invasion by the primary tumor of the surrounding host tissues, followed by intravasation and dissemination of the tumors cells via the blood or lymphatic system, infiltration and colonization at the distant site. Several factors and pathways are known to influence cancer metastasis including disintegration of cell-to-cell adhesion, proteolytic remodeling of the extracellular matrix, cell motility, evasion of immune and apoptotic signals and angiogenesis. Although substantial information is known about the process of metastasis, the molecular basis of breast cancer metastasis remains poorly understood. NRs, being master regulators of almost every physiological aspect of life, are likely to be intimately involved in the metastasis process. Here, we summarize the current understanding of the roles of various NRs in biological pathways affecting breast cancer metastasis.

Estrogen receptors The estrogen receptors alpha (ESR1) and beta (ESR2) appear to have distinct roles in breast cancer progression and metastasis, with reports of ESR1 signaling enhancing breast cancer cell migration and invasion, whereas ESR2 signaling has the opposite effects.

Emerging evidence suggests that estradiol's positive effect on cell migration and invasion appears to be mediated through ESR1's extranuclear signaling. ESR1 extranuclear signaling is mediated through the PI3K, MAPK and c-Jun N-terminal kinase pathways (Li *et al.* 2010, Zheng *et al.* 2011) with the involvement of PELP1 (Chakravarty *et al.* 2010), focal adhesion kinase (FAK)

(Sanchez *et al.* 2010), c-Src and paxillin (Li *et al.* 2010) for activation. Downstream activation of the RhoA/ROCK-2 cascade has also been reported (Giretti *et al.* 2008, Zheng *et al.* 2011). Recruitment of the actin-binding protein moesin (Giretti *et al.* 2008) and phosphorylation of the actin-binding protein ezrin (Zheng *et al.* 2011) were reported as possible mechanisms through which ESR1 signaling influences the cytoskeletal organization. These E2-mediated signaling pathways induce features of motile cells such as dynamic actin cytoskeleton remodeling and formation of ruffles and filopodia-like structures, which resulted in enhanced motility and migration of breast cancer cells (Chakravarty *et al.* 2010).

ESR2, on the other hand, has been associated with less invasive and proliferative tumors (Jarvinen *et al.* 2000, Lazennec *et al.* 2001). Overexpression of ESR2 leads to reduced cell growth and mobility (Li *et al.* 2012) and ESR2 was reported to upregulate E-cadherin (Zhou *et al.* 2015), integrin alpha1, integrin beta1 and enhance the formation of vinculin-containing focal complexes and actin filaments (Lindberg *et al.* 2010), which consequently strengthens cell adhesion and reduces migration and invasion.

However, ESR2's influence on cell migration is possibly more complex than simply having an anti-migratory and anti-proliferative effect, with contradictory reports of ESR2 having both pro-invasive and pro-migratory effects, especially in specific breast cancer subtypes, due to crosstalk with ESR1 as well as differential effect of different ESR2 variants. ESR2 and PEA3 were reported to co-activate IL-8 resulting in increased invasiveness in ER+ MCF-7 cells (Chen *et al.* 2011). In inflammatory breast cancer cells, which express both ESR2 and ESR1₃₆ (an ESR1 variant), it was suggested that non-genomic signaling involving ESR1₃₆, ESR2 and GPR30 through activation of p-ERK1/2 to have pro-migratory and pro-invasive effect (Ohshiro *et al.* 2012). Furthermore, expression of an ESR2 variant, ESR2cx in primary tumors, was reported to correlate with higher risk of lymph node metastasis (Rosin *et al.* 2014). In summary, the effects of estrogen receptor signaling on breast cancer cell metastasis is complex with different isoforms having opposing roles, which is further complicated by divergent effects of subtype variants and crosstalk. The emerging picture is that of ESR1 exerting pro-migratory and pro-invasive effects through extranuclear signaling to regulate cell adhesion and cytoskeletal remodeling while ESR2 exerts the opposite effect through as yet unclear mechanisms.

Progesterone receptors PGR in the human is expressed as two functionally different forms (PGRA and PGRB) arising from alternate promoter activities driving a single gene (Kastner *et al.* 1990). PGRA and PGRB are equivalently expressed in most normal cells, but commonly their expression becomes deregulated in breast cancer resulting in a predominance of one isoform (usually PGRA) (Graham *et al.* 1995). This disruption is seen early in disease progression (Mote *et al.* 2002) and is associated with a poorer response to the anti-estrogen tamoxifen (Mote *et al.* 2015). A number of studies have demonstrated progestin effects on breast cancer cell adhesion, migration and invasive potential. However, these effects are ligand, PGR isoform and context dependent. In general, progestins acting through nuclear PGR inhibit cell migration and invasiveness (Lin *et al.* 2000, 2001, McGowan *et al.* 2004, Carnevale *et al.* 2007). In cells transfected with PGR, progestin treatment increases actin stress fibers and focal contacts, consistent with increased adhesion and decreased migration (Lin *et al.* 2000, 2001). Invasion of T-47D breast cancer cells, expressing equivalent PGR isoform levels, is inhibited by progestins (McGowan *et al.* 2004). However, predominance of PGRA results in an altered progestin response, including increased focal adhesion signaling (Graham *et al.* 2005), enhanced migration and increased invasion (McGowan *et al.* 2004), potentially contributing to the poorer outcomes seen in PGRA-predominant breast cancers.

Crosstalk between PGR and cytoplasmic signaling pathways is implicated in breast cancer migration and metastasis. Using a DNA-binding mutant PGR, Carnevale and coworkers (Carnevale *et al.* 2007) reported that PGR activated cytoplasmic signaling cascades that contributed to breast cancer metastasis. Progestin activation of MAPK, PI3K/Akt and GTPase/RhoA modulated the expression of genes such as urokinase plasminogen activator (uPA), uPA receptor (uPAR), matrix metalloproteases (MMPs), β 1-integrin and PAI-1 (Carnevale *et al.* 2007, Fu *et al.* 2008, Bellance *et al.* 2013) suggesting a mechanism through which migration is increased by progestins. In addition, progesterone/progestins also enhance the phosphorylation of focal adhesion kinase (FAK) (Graham *et al.* 2005, Fu *et al.* 2008, Bellance *et al.* 2013), and this is enhanced with PGRA predominance (Graham *et al.* 2005), promoting the formation and turnover of focal adhesion points and cytoskeletal reorganization involved in cellular motility and influencing breast cancer progression and metastasis (Luo & Guan 2010).

PGR has also been reported to exert ligand-independent effects on breast cancer cell morphology and migration (Jacobsen *et al.* 2005, Carnevale *et al.* 2007, Bellance *et al.* 2013). Un-liganded PGRA was shown to differentially regulate genes involved in cell adhesion and increase adhesiveness and migration in inducible cells expressing just one isoform (Jacobsen *et al.* 2005). However, in a recent study using bi-inducible MDA-MB-231 cells, un-liganded PGRB but not PGRA increased cell migration via differential effects on focal adhesion signaling (Bellance *et al.* 2013). Furthermore, it has been demonstrated that extensive ligand-independent action of PGRB are dependent on specific post-translational modifications, particularly phosphorylation, and associated with pro-survival pathways (Daniel *et al.* 2007, Faivre *et al.* 2008, Knutson *et al.* 2012). These findings in the absence of ligand suggest that disrupted PGR isoform expression may have particular importance in the context of post-menopausal breast cancer, where circulating progesterone is low or absent.

In summary, the emerging picture is that the effects of PGR and progesterone on breast cancer metastasis, adhesion and invasion are context dependent and influenced by the relative ratio of PGR isoforms being expressed, the presence of ligands and whether PGR functions as a transcription factor or activator of signaling cascades, but that PGR contributes to increased invasive and metastatic behavior in a number of contexts.

Retinoic acid receptors Several studies demonstrated the anti-proliferative and anti-migratory effect of retinoids on breast cancer cells. Specifically, all-trans retinoid acid (ATRA) was reported to modulate several migration-related proteins such as downregulation of MMP-1, MMP-2, MMP-9, FAK, NF- κ B and p-ERK and upregulation of E-cadherin in breast cancer cell lines (Liu *et al.* 2003, Dutta *et al.* 2009, 2010). The anti-migratory effect of retinoids on breast cancer cells were shown to be mediated through RARB with treatment of RA or RARB agonist resulting in significantly reduced cell migration while treatment with RARA or RARG agonists did not (Flamini *et al.* 2014). RARB is the only member of the RARs showing decreased expression in breast cancers (Muscat *et al.* 2013). RARB showed progressively decreased expression during breast carcinogenesis, with only around 50% of invasive breast carcinoma expressing RARB (Xu *et al.* 1997). Transduction of RARB2 resulted in significant reduction in metastasis rate in a mouse xenograft model (Treuting *et al.* 2002). Together, these

findings highlight that RARB is necessary for mediating the anti-migratory effect of retinoic acid through modulation of expression of genes involved in cell migration.

Estrogen-related receptors Aside from their prominent role as master regulators of energy metabolism, the ERRs have been reported to influence the migratory potential of breast cancer cells. Knockdown of ESRRa dramatically decreased the migratory potential of breast cancer cells (Stein *et al.* 2008), later determined to be mediated through VEGF (Stein *et al.* 2009) and WNT11 (Dwyer *et al.* 2010) signaling pathways, which are known regulators of angiogenesis. Inactivation of ESRRa also resulted in impaired directional migration of breast cancer cells, mediated through enhanced stability of RHOA, a protein involved in controlling oriented cellular migration (Sailland *et al.* 2014). ESRRa itself was found to be a target of miR-137 (Zhao *et al.* 2012). Therefore, consistent with reports of its association with poor prognosis, activation of ESRRa has a pro-migratory effect on breast cancer cells. By contrast, and consistent with its association with better breast cancer outcome, ESRRg was reported to promote mesenchymal-to-epithelial transition and decrease breast cancer cell invasiveness through activation of E-cadherin (Tiraby *et al.* 2011). Therefore, similar to their opposing roles in metabolic reprogramming, ESRRa and ESRRg display opposing influences on breast cancer cell migration and invasiveness.

NUR77 NUR77 (NR4A1) expression is elevated in both ER+ and ER- breast tumors (Muscat *et al.* 2013), and NUR77 expression is correlated with decreased relapse-free survival in breast cancer (Alexopoulou *et al.* 2010), consistent with reports of its pro-oncogenic role in breast cancer (Hedrick *et al.* 2015). Contradictory to a previous report of NUR77 having anti-migratory effects in MCF-10A breast cancer cells, recently, NUR77 expression was shown to have pro-migratory effects through activation of TGF-beta signaling (Zhou *et al.* 2014), which is known to have an important role in breast cancer metastasis (Moore *et al.* 2008, Kohn *et al.* 2012, Luwor *et al.* 2015).

COUP-TF1 and COUP-TF2 COUP-TFs are orphan nuclear receptors primarily known for their important roles in organogenesis including angiogenesis, cellular growth, differentiation and migration (Boudot *et al.* 2011). Their roles in breast cancer growth and progression have recently been highlighted. Both COUP-TF1 and COUP-TF2 expression are lower in breast tumors

compared to normal breast tissue (Muscat *et al.* 2013). *COUP-TF1* expression was reported to be higher in dedifferentiated breast cancer cells and correlated with low expression of E-cadherin and expression of vimentin (Le Dily *et al.* 2008). Overexpression of *COUP-TF1* in MCF-7 cells results in a decrease in *CXCL12* and increase in *CXCR4* expression, mediated by EGF signaling, and leads to enhanced motility and invasiveness of MCF-7 breast cancer cells (Le Dily *et al.* 2008, Boudot *et al.* 2014). *CXCL12* expression in primary tumors has recently been reported to drive breast cancer metastasis (Ray *et al.* 2015). The other COUP-TF member, COUP-TF2, may also have a role in breast cancer cell migration and invasion with a report that transfection of COUP-TF2 into MDA-MB-231 resulted in increased migration and invasion of these cells (Navab *et al.* 2004). Together these findings suggest a potential involvement of COUP-TFs in epithelial-to-mesenchymal transition and migration of breast cancer cells.

Concluding remarks

Advances in genomic technologies have enabled more integrated insights into the roles of NRs in breast cancer biology: their expression, genomic distribution, mechanisms of action, as well as the complexity of their functional interactions. Expression profiling studies have shown that many NRs, in addition to ESR1 and PGR, are expressed and are potentially functional in breast cancer. Cistromic profiling studies highlight the complexity of NR transcriptional regulation, how multiple NRs often work together as well as with other coregulators and the chromatin landscape to fine tune the cell-specific expression of target genes, and how changes in the gene regulatory programs of NR are associated with the tumor state. Finally, it is evident that in addition to regulating proliferation and apoptosis, NRs are critical regulators of a diverse range of other cancer-associated biological processes including the circadian clock, metabolic reprogramming and migration and metastasis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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